

Comparison of proton-specific ATPase activities in plume and root tissues of two co-occurring hydrocarbon seep tubeworm species *Lamellibrachia luymesii* and *Seepiophila jonesi*

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Abstract *Lamellibrachia luymesii* and *Seepiophila jonesi* are co-occurring species of vestimentiferan tubeworms found at hydrocarbon seepage sites on the upper Louisiana slope of the Gulf of Mexico. Like all vestimentiferans, they rely on internal sulfide-oxidizing symbiotic bacteria for nutrition. These symbionts produce hydrogen ions as a byproduct of sulfide oxidation, which the host tubeworm needs to eliminate to prevent acidosis. The hydrothermal vent tubeworm *Riftia pachyptila* uses a high activity of P- and V-type H⁺-ATPases located in its plume epithelium to excrete protons. Unlike *R. pachyptila*, the seep species grow a posterior root, which they can use in addition to their plumes as a nutrient exchange surface. In this study we measured the ATPase activities of plume and root tissues collected from *L. luymesii* and *S. jonesi*, and used a combination of inhibitors to determine the relative activities of P- and V-type H⁺-ATPases. We found that the total H⁺-ATPase activity of their plumes was approximately 14 $\mu\text{mol h}^{-1} \text{g}^{-1}$ wet weight, and that of their roots was between 5 and 7 $\mu\text{mol h}^{-1} \text{g}^{-1}$ wet weight. These activities were more than ten times lower than those measured in *R. pachyptila*. We suggest that seep tubeworms might use passive channels to eliminate protons across their roots, in

addition to ATP-dependant proton pumps located in their plumes and roots. In addition, we found strong differences between the types of ATPase activities in the plumes of *L. luymesii* and *S. jonesi*. While the H⁺-ATPase activity of *L. luymesii* plumes is dominated by P-type ATPases, *S. jonesi* has an unusually high activity of V-type H⁺-ATPases. We suggest that *S. jonesi* relies on its high V-type H⁺-ATPase activity to drive carbon dioxide uptake across its plume surface. *L. luymesii*, on the other hand, might rely partially on bicarbonate uptake across its root.

Introduction

All living organisms maintain ionic homeostasis using membrane transport processes. Transport ATPases, which couple the energy derived from ATP hydrolysis to drive transport of solutes against their electrochemical gradients, are a ubiquitous type of membrane transport protein (Pedersen 1982). They are generally of three different categories: P-, V-, and F-type ATPases (Van Winkle 1999). P-type ATPases, named so because they are temporarily phosphorylated during their transport cycle, mediate transport of several different inorganic ions, including sodium, potassium, hydrogen, and calcium ions (Møller et al. 1996). The plasma membrane Na⁺/K⁺-ATPase is an example of a P-type ATPase. F- and V-type ATPases have multiple domains, and nearly all of them transport protons (Nelson 1992). In multicellular organisms, V-type ATPases are usually found in endomembranes and plasma membranes, whereas F-type ATPases are typically located in inner mitochondrial or thylakoid membranes (Bowman et al. 1988; Wieczorek et al. 2000).

Compared to other marine invertebrates, the hydrothermal vent vestimentiferan tubeworm *Riftia pachyptila*

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(Phylum Annelida, Class Polychaete, Family Siboglinidae) has unusually high ATPase activity, with a large proportion of its ATPases being P- and V-type ATPases devoted to proton transport (Goffredi and Childress 2001). *R. pachyptila* obtains sulfide, carbon dioxide, and oxygen from its environment and supplies them to internal sulfide-oxidizing bacterial symbionts, which it relies on for nutrition (Childress et al. 1984, 1991; Arp et al. 1985). The symbionts produce sulfate and hydrogen ions as end products of sulfide oxidation (Childress et al. 1984, 1991; Childress and Fisher 1992). *R. pachyptila* uses its highly vascularized plume for sulfide uptake, as well as sulfate and proton elimination (Arp et al. 1985; Goffredi et al. 1998). Since electrochemical gradients are unfavorable for both sulfate and proton elimination across the plume into the surrounding seawater, *R. pachyptila* expends a substantial amount of energy for eliminating these ions, and uses the high concentration of proton-specific ATPases located on its plume for proton excretion (Goffredi et al. 1998; Goffredi and Childress 2001; Girguis et al. 2002).

The vestimentiferans *Lamellibrachia luymesii* and *Seepiophila jonesi* normally co-exist in aggregations at hydrocarbon seep sites in the Gulf of Mexico (Bergquist et al. 2002). Unlike *R. pachyptila*, *L. luymesii* and *S. jonesi* grow root-like posterior extensions of their body, which they could potentially use as a metabolite-exchange surface in addition to their plume. To date, most physiological studies on hydrocarbon seep tubeworms from the Gulf of Mexico have focused on *L. luymesii*. These studies have established that *L. luymesii* uses its roots for sulfide uptake, as well as for elimination of sulfate and hydrogen ions (Julian et al. 1999; Freytag et al. 2001; Dattagupta et al. 2006). Although knowledge about *S. jonesi*'s physiology is limited, circumstantial evidence suggests that relative to *L. luymesii*, it might rely more on its plume than its root for metabolite-exchange. While adult *L. luymesii* plumes are often over a meter above the sediment surface, where sulfide levels are undetectable (lower than 0.1 μM), adult *S. jonesi* plumes are normally close to the sediment surface where sulfide at low micromolar levels can often be detected (Freytag et al. 2001; Bergquist et al. 2003). Since *S. jonesi* hemoglobins have substantially higher affinity for sulfide than *L. luymesii* hemoglobins, *S. jonesi* could potentially acquire a substantial proportion of its sulfide across its plume (Freytag et al. 2003). Moreover, the chitin tubes surrounding *S. jonesi* roots are significantly less permeable than those of *L. luymesii* (K.E. Luley, unpublished data), suggesting that *S. jonesi* might not rely on its root as a significant metabolite-exchange surface.

In this study, we characterized the in vitro ATPase activities of plume and root tissues collected from *L. luymesii* and *S. jonesi*. Using inhibitors that target specific types of ATPases, we estimated the proton-specific ATPase activities

of these tissues. In a sequential series of experiments, we used ouabagenin to inhibit Na^+/K^+ -ATPases, vanadate to inhibit P-type ATPases, *N*-ethylmaleimide to inhibit P- and V-type ATPases, azide to inhibit F-type ATPases, and bafilomycin to inhibit V-type ATPases (Bowman et al. 1988; Lin and Randall 1993; Goffredi and Childress 2001). Based on the hypothesis that *S. jonesi* relies more heavily on its plume as a metabolite-exchange surface than *L. luymesii*, we predicted lower proton-specific ATPase activities in *L. luymesii* plumes than *S. jonesi* plumes. Moreover, roots of *L. luymesii* and *S. jonesi* are buried in sediment where microbial sulfate reduction depletes protons, and calculations based on electrochemical gradients suggest that these tubeworms could use passive proton transport across their roots instead of ATPase mediated proton transport (Arvidson et al. 2004; Dattagupta et al. 2006). Based on this, we predicted relatively low proton-specific ATPase activities in *L. luymesii* and *S. jonesi* root tissues.

Materials and methods

Collection of tissues for analysis

L. luymesii and *S. jonesi* were collected from a site in the Mineral Management Service lease block GC234, located at a depth of approximately 540 m on the upper Louisiana slope of the Gulf of Mexico (27°44.7'N, 91°13.3'W; MacDonald et al. 1990), using the robotic manipulator of the Johnson Sea-Link submersible. The tubeworms were transported to the surface in a temperature-insulating box, and maintained in cold seawater for 2–3 h prior to dissection. Plume and root tissues (not separated from trophosome) were obtained and frozen using liquid nitrogen within 30 min after the dissection was complete. They were transported in liquid nitrogen to the laboratory at Penn State University, where they were kept frozen at -70°C until further analysis.

Preparation of tissue homogenates

Homogenization and subsequent ATPase assays were performed using modifications of procedures described previously (Lin and Randall 1993; Goffredi and Childress 2001). Frozen tissues were thawed on ice. Plumes were dissected to separate gill tissue from obturaculum, and root body wall was carefully separated from trophosome. The tissues were weighed and then homogenized on ice in 30 μl mg^{-1} of tissue homogenization buffer to make a crude homogenate (hereafter designated 'C'). Gill tissue was homogenized using a mechanical tissue homogenizer (Polytron PT 3,000, Brinkmann Instruments, Inc.), whereas root wall was homogenized using a Pyrex glass homogenizer. The

homogenization buffer (pH adjusted to 7.3) contained 50 mM imidazole hydrochloride, 20 mM ethylenediamine tetraacetic acid (EDTA), 300 mM sucrose, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), and 5 mM β -mercaptoethanol (bME) (Goffredi and Childress 2001). A 50 μ l aliquot of the crude homogenate was stored at -70°C until it was used for ATPase assays, and the remaining homogenate was centrifuged at 2,000g for 7 min, with the intention of enriching mitochondria in the supernatant, and the cell membrane fraction in the pellet (Lin and Randall 1993). The supernatant (hereafter designated ‘S1’) was stored at -70°C until further analysis. The pellet was dissolved in 10 \times volume homogenization buffer containing 6% 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) zwitterionic detergent, in order to solubilize membrane proteins. It was then centrifuged at 2,000g for another 7 min, to enrich cell membrane proteins in the supernatant, and cell debris in the pellet (Lin and Randall 1993). The supernatant (S2) was divided into 100 μ l aliquots and stored at -70°C until it was used for ATPase assays.

Determination of mitochondrial content: succinate dehydrogenase activity

The activity of the mitochondrial marker enzyme succinate dehydrogenase (SDH) was determined in homogenate fractions C, S1, and S2 as previously described (Munujos et al. 1993). This assay is based on the conversion of 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT), a colorless compound, to formazan, a blue compound with a light absorption peak at 500 nm. To perform the assay, 10 μ l of the homogenate was mixed with 990 μ l of the assay buffer (pH adjusted to 8.3) that contained 100 mM Tris hydrochloride, 0.5 mM EDTA, 2 mM potassium cyanide, 2 mM INT, 20 mM succinate (pH adjusted to 7.4 before it was added to the assay medium), and 12 g/l cremophor EL. Absorbance at 500 nm was measured every 30 s for 6 min, and the mitochondrial activity was determined by subtracting absorbance of a control containing all assay components except for succinate.

Determination of plasma membrane content in fractions: Na^+/K^+ -ATPase activity

The activity of the plasma membrane marker enzyme Na^+/K^+ -ATPase was determined in fractions C, S1, and S2. Na^+/K^+ -ATPase activity of fractions is usually estimated by determining ouabain-sensitive ATPase activity (Furriel et al. 2001). However, Na^+/K^+ -ATPases of vestimentiferan tubeworms appear to be insensitive to ouabain, but sensitive to its analogue, ouabagenin (Goffredi and Childress 2001). Therefore, we estimated Na^+/K^+ -ATPase activity of

fractions by determining ouabagenin-sensitive ATPase activity using the methodology described below.

Measurement of ATPase activity

ATPase activity was determined by spectrophotometric assay that uses a coupled pyruvate kinase/lactate dehydrogenase enzyme reaction (Lin and Randall 1993; Goffredi and Childress 2001). The assay medium (pH 7.3) was prepared fresh daily and contained 50 μ M fructose diphosphate, 2 mM phosphoenol pyruvate (PEP), 3 mM adenosine-5'-triphosphate (ATP), 0.2 mM reduced β -nicotinamide adenine dinucleotide (NADH), 12.7 units/ μ l lactate dehydrogenase, and 15 units/ μ l pyruvate kinase, made in a stock solution containing 100 mM sodium chloride, 20 mM potassium chloride, 5 mM magnesium chloride, 30 mM imidazole, and 0.5 mM ethylene glycol bis-(2-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA). One hundred microliters of the tissue homogenate was mixed with 900 μ l of assay medium and readings were taken at 340 nm every 30 s over a period of 5–10 min using a Beckman DU-64 spectrophotometer (Beckman Coulter Inc., Fullerton, CA, USA), equipped with a temperature controlled cuvette (15°C). Protein content of the homogenates was determined using the Bradford method, using a 1 mg/ml bovine serum albumin (BSA) standard prepared in the homogenization buffer. ATPase activity was expressed in either protein-specific inorganic phosphate values ($\mu\text{mol P}_i \text{ h}^{-1} \text{ mg}^{-1}$ protein) or tissue wet weight specific values ($\mu\text{mol P}_i \text{ h}^{-1} \text{ g}^{-1}$ wet weight).

Inhibitor exposure

Tissue homogenates were incubated with various ATPase-specific inhibitors before the assay was conducted. Fifty microliters of a stock solution of an inhibitor (or solvent, in the case of controls) was mixed with 50 μ l of the tissue homogenate. The mixture was incubated at room temperature for a period of 2 h, with the exception of Bafilomycin, which was incubated for 30 min (Goffredi and Childress 2001). The inhibitor stock solutions were: 20 mM sodium azide, 40 mM ouabagenin, 20 mM NEM, 20 mM vanadate, and 7 μ M bafilomycin. Bafilomycin stock solution was made using 5% DMSO as solvent, and corresponding controls contained the same amount of DMSO. All other inhibitor solutions were made using water. Azide, vanadate, and bafilomycin stock solutions were made in advance and the first two were stored at room temperature, whereas bafilomycin was stored at -20°C . The vanadate stock solution was monitored at 400 nm to ensure that decameric vanadium species were negligible or absent in the solution (Aureliano and Gândara 2005). The NEM stock solution was made fresh daily.

Estimation of proton-specific P- and V-type ATPase activities

Inhibitor sensitivity was used to estimate proton-specific ATPase activities as described below. At the concentrations used in our assays, NEM inhibits both P- and V-type ATPases (Pedersen and Carafoli 1987; Forgac 1989). Since vanadate sensitivity was found to be low in most tissues examined, NEM-sensitivity was used to estimate the total P- and V-type ATPase activity. Bafilomycin-sensitivity was used to estimate V-type ATPase activity, as it is extremely specific to V-type ATPases at concentrations used in our experiments (Bowman et al. 1988). Since EGTA, a Ca^{2+} -chelator was used in our assays, Ca^{2+} -ATPase activity was assumed to be negligible (Lin and Randall 1993; Goffredi and Childress 2001). Total P-type ATPase activity, which includes Na^+/K^+ -ATPases, and H^+ -ATPases, was estimated by subtracting bafilomycin-sensitive activity from NEM-sensitive activity. Finally, P-type H^+ -ATPase activity was estimated by subtracting ouabagenin-sensitive Na^+/K^+ -ATPase activity from the total P-type ATPase activity.

Results

Mitochondrial activity of fractions

SDH activity, which was used to estimate mitochondrial content, was highest in the crude homogenates (C) of all tissues examined (Fig. 1). The S1 supernatant fraction contained between 26 and 77% of the total SDH activity, indicating substantial removal of mitochondria using the first centrifugation step. However, the fraction S2, which was subsequently used to analyze total ATPase activity, still showed some SDH activity. The S2 fractions of *L. luymesi* and *S. jonesi* plume tissues contained, respectively, 25 and 22% of the mitochondrial activity found in their respective crude homogenates. On the other hand, the S2 fractions of

L. luymesi and *S. jonesi* root tissues contained, respectively, 0 and 5% of the total mitochondrial activity. Therefore, mitochondrial removal was more efficient in the root than the plume tissue homogenates.

Na^+/K^+ -ATPase activity of fractions

Ouabagenin-sensitive activity of fractions, which was used to estimate activity of the plasma membrane marker enzyme Na^+/K^+ -ATPase, indicated that the purification process was successful in enriching plasma membrane in the S2 fraction (Fig. 2). Using the mean values of the Na^+/K^+ -ATPase activities, we estimated that the enrichment of plasma membrane in the S2 fraction was 288 and 168%, respectively, for *L. luymesi* plume and root tissues. Similarly, the enrichment was 159 and 125%, respectively, for *S. jonesi* plume and root tissues.

Total ATPase activities

Total ATPase activity in $\mu\text{mol P}_i \text{ h}^{-1} \text{ mg}^{-1}$ protein was similar in all tissues examined (Table 1; $P > 0.05$; two-sided t test). However, both species had significantly higher protein content, i.e., mg protein per g tissue wet weight (WW), in plume than in root tissues ($P < 0.0025$; two-sided t tests). As a result, wet weight specific plume ATPase activities ($\mu\text{mol P}_i \text{ h}^{-1} \text{ g}^{-1}$ WW) were significantly higher than root ATPase activities in both species. The average *S. jonesi* plume and root ATPase activities ($\mu\text{mol P}_i \text{ h}^{-1} \text{ g}^{-1}$ WW) were higher than *L. luymesi* plume and root ATPase activities, but the differences were not significant (plume: $P = 0.246$; root: $P = 0.232$; two-sided t tests).

Inhibitor sensitivities

Inhibitor studies were performed with *L. luymesi* and *S. jonesi* plume and root tissues, and comparisons were made between species and tissue types (Fig. 3). For *L. luymesi*,

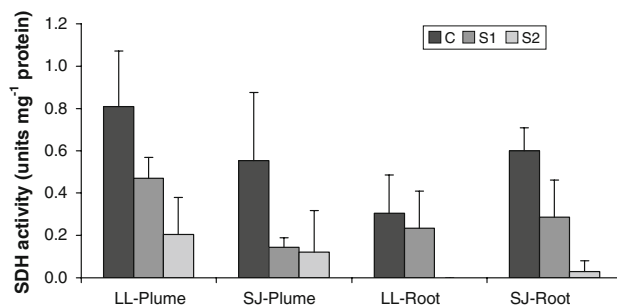


Fig. 1 Mean \pm standard deviation of succinate dehydrogenase (SDH) activity in the crude, S1 and S2 fractions of the various tissues analyzed ($N = 5$ for all tissues). LL and SJ correspond to *L. luymesi* and *S. jonesi*, respectively

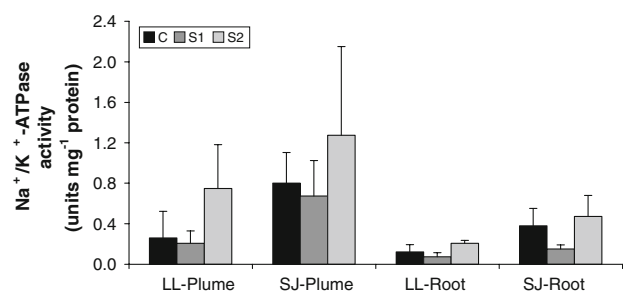
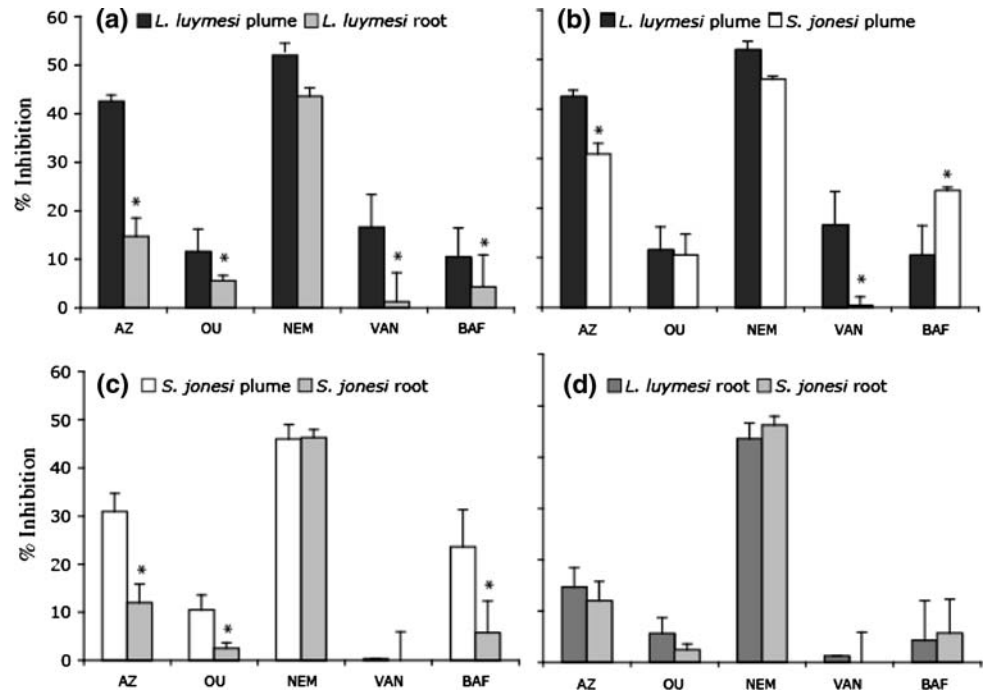


Fig. 2 Mean \pm standard deviation of Na^+/K^+ -ATPase activity in the crude, S1 and S2 fractions of the various tissues analyzed ($N = 4$ for plume tissues; $N = 3$ for root tissues). LL and SJ correspond to *L. luymesi* and *S. jonesi*, respectively

Table 1 The total ATPase activity of various tissues examined in this study

Species	Plume ATPase activity		Root ATPase activity	
	$\mu\text{mol P}_i \text{ h}^{-1} \text{ mg}^{-1}$ protein	$\mu\text{mol P}_i \text{ h}^{-1} \text{ g}^{-1}$ WW	$\mu\text{mol P}_i \text{ h}^{-1} \text{ mg}^{-1}$ protein	$\mu\text{mol P}_i \text{ h}^{-1} \text{ g}^{-1}$ WW
<i>L. luymesii</i> (N = 15)	2.8 ± 1.3	34.9 ± 14.6	2.6 ± 1.4	13.1 ± 6.5
<i>S. jonesii</i> (N = 12)	2.0 ± 0.6	41.4 ± 13.4	2.5 ± 0.7	16.3 ± 7.6

Fig. 3 Comparison of percent inhibition of total ATPase activity by various inhibitors in **a** *L. luymesii* plume and root tissues; **b** *L. luymesii* and *S. jonesii* plume tissues; **c** *S. jonesii* plume and root tissues; and **d** *L. luymesii* and *S. jonesii* root tissues. An asterisk implies a significant difference ($P < 0.05$; two-sided t test). AZ azide, OU ouabagenin, NEM *N*-ethylmaleimide, VAN vanadate, BAF bafilomycin. Percent inhibition is expressed with respect to baseline activity measured in the presence of the solvent used to make inhibitor solutions

percent inhibition by all inhibitors was higher for plume than for root tissues, and this difference was significant ($P < 0.05$; two-sided t test) for all inhibitors except NEM. Similarly, percent inhibition by azide, ouabagenin, and bafilomycin were significantly higher for *S. jonesii* plume than for root tissues. The higher inhibition by azide in plume tissues of both species was consistent with the higher mitochondrial content found in their S2 fractions (Fig. 1). *L. luymesii* and *S. jonesii* root tissues were very similar with respect to inhibition by all compounds tested, whereas there were some notable differences between their plume tissues. While azide and vanadate inhibition were significantly higher for *L. luymesii* plume tissue, bafilomycin inhibition was significantly higher for *S. jonesii* plume tissue. Except for *L. luymesii* plume tissue, all other tissues appeared to be insensitive to vanadate inhibition.

We calculated activities of the V- and the P-type H^+ -ATPase for the root and plume tissues of both species based on the results from the inhibitor experiments (Table 2). Although the total ATPase activity is very similar for a given tissue between species, the contribution of V- and P-type is very different in the plumes. Most of the activity in

S. jonesii plume is due to V-type H^+ -ATPase while most of the activity in *L. luymesii* plume is due to P-type H^+ -ATPase.

Discussion

Seep tubeworms have much lower ATPase activities than *R. pachyptila*

R. pachyptila, the vestimentiferan tubeworm found at hydrothermal vent sites on the East Pacific Rise, has protein-specific ATPase activities that are approximately four times higher than that of other marine invertebrates that do not harbor symbionts (Goffredi and Childress 2001). Using an inhibitor study similar to ours, the authors concluded that a majority of *R. pachyptila*'s ATPase activity was used for elimination of protons produced by its symbionts during sulfide oxidation. In contrast, we found that the protein-specific ATPase activities of the two hydrocarbon seep tubeworm species, *L. luymesii* and *S. jonesii*, were similar to that of non-symbiotic, shallow-living marine invertebrates investigated by Goffredi and Childress (2001).

Table 2 Estimated P- and V-type ATPase activities (in $\mu\text{mol P}_i \text{ h}^{-1} \text{ g}^{-1}$ tissue wet weight) of *L. luymesii* plume (LP), *L. luymesii* root (LR), *S. jonesi* plume (SP), and *S. jonesi* root (SR)

ATPase type	Inhibitor sensitivity	ATPase activity ($\mu\text{mol P}_i \text{ h}^{-1} \text{ g}^{-1}$ WW)			
		LP	LR	SP	SR
Total	None	35.0	13.1	41.4	16.3
Total P- and V-type ATPase	NEM	18.2	5.8	19.0	7.5
V-type H^+ ATPase	Bafilomycin	3.7	0.6	9.8	0.9
Total P-type ATPase	NEM- (Bafilomycin)	14.5	5.2	9.3	6.6
Na^+/K^+ -ATPase	Ouabagenin	4.1	0.7	4.3	0.4
P-type H^+-ATPase	NEM- (Bafilomycin + Ouabagenin)	10.5	4.5	4.9	6.2

Proton-specific ATPase activities are in bold

Like *R. pachyptila*, *L. luymesii* and *S. jonesi* have sulfide-oxidizing symbionts that produce protons as a byproduct of their metabolism. However, seep tubeworms have lower metabolic rates than *R. pachyptila*, which could explain the lower ATPase activities we measured. Alternately, seep tubeworms might rely on passive transport of waste ions such as sulfate and protons across their root surface (Dattagupta et al. 2006), diminishing their need for high tissue ATPase activities, as discussed below.

L. luymesii might use proton channels in its roots for proton elimination

An important distinction between *R. pachyptila* and the seep tubeworms we examined is the presence of posterior buried roots in the latter species. Both *L. luymesii* and *S. jonesi* have long posterior roots, but so far, the use of roots for metabolite-exchange has been better studied in *L. luymesii* than in *S. jonesi*. Based on electrochemical gradients, proton movement across *L. luymesii*'s plume epithelium into the external seawater medium would require active transport, whereas it could occur passively across the root epithelium (Dattagupta et al. 2006). In this study, we found that *L. luymesii* plume tissue has a total H^+ -ATPase activity of $14 \mu\text{mol P}_i \text{ h}^{-1} \text{ g}^{-1}$ wet weight, whereas its root tissue has an activity of only $5 \mu\text{mol P}_i \text{ h}^{-1} \text{ g}^{-1}$ wet weight (Table 2). Given the fact that *L. luymesii* eliminates a majority of its protons across its roots (Dattagupta et al. 2006), it appears that proton channels, rather than H^+ -ATPases might mediate proton transport across *L. luymesii*'s root epithelium.

In laboratory experiments where *L. luymesii* and *R. pachyptila* were exposed to similar levels of external sulfide, *L. luymesii*'s plume proton elimination rates were on average $11.9 \mu\text{equivalents g}^{-1} \text{ h}^{-1}$, compared to *R. pachyptila*'s elimination rate of $40.5 \mu\text{equivalents g}^{-1} \text{ h}^{-1}$ (Girguis et al. 2002). Since *L. luymesii* eliminates approximately 67% of its protons across its roots (Dattagupta et al. 2006), the results of these studies indicate that the total

proton elimination rate of *L. luymesii* is approximately $36 \mu\text{equivalents g}^{-1} \text{ h}^{-1}$, slightly lower than the mass-specific rate of *R. pachyptila*. However, the mass-specific H^+ -ATPase activity of *L. luymesii* is more than ten times lower than that of *R. pachyptila*. *R. pachyptila* has a plume P-type H^+ -ATPase activity of $136\text{--}174 \mu\text{mol P}_i \text{ h}^{-1} \text{ g}^{-1}$ wet weight and a V-type H^+ -ATPase activity of $80 \mu\text{mol P}_i \text{ h}^{-1} \text{ g}^{-1}$ wet weight (Goffredi and Childress 2001). On the other hand, *L. luymesii* has a total (plume and root) P-type H^+ -ATPase activity of $15 \mu\text{mol P}_i \text{ h}^{-1} \text{ g}^{-1}$ wet weight and a V-type H^+ -ATPase activity of $4 \mu\text{mol P}_i \text{ h}^{-1} \text{ g}^{-1}$ wet weight (Table 2). The large discrepancy between these values is consistent with *L. luymesii* relying partly on passive proton channels for proton elimination across its root epithelium, in addition to H^+ -ATPases in its plume and root tissues.

The pH of *S. jonesi* body fluids is similar to that of *L. luymesii* (S. Dattagupta, unpublished data) and the two seep species commonly co-exist in aggregations (Bergquist et al. 2002). Since *S. jonesi* also has roots buried in sediments where pore water pH is depleted, it could potentially eliminate protons using passive transport across its roots. In this study, we found that its plume H^+ -ATPase activity was $14.7 \mu\text{mol P}_i \text{ h}^{-1} \text{ g}^{-1}$ wet weight, whereas its root H^+ -ATPase activity was $7.1 \mu\text{mol P}_i \text{ h}^{-1} \text{ g}^{-1}$ wet weight. While it appears likely that *S. jonesi* use their roots for passive proton transport, laboratory experiments that directly measure its proton elimination rates are necessary to substantiate this possibility.

The relative abundance of P- and V-type ATPases varies between seep species

Based on differences in growth habits of *L. luymesii* and *S. jonesi*, we predicted that *S. jonesi* would rely more on its plume for metabolite-exchange than *L. luymesii* does. Contrary to our expectations, we found very similar total plume H^+ -ATPase activities in the two tubeworm species. However, there were some interesting differences in the relative abundances of P- and V-type H^+ -ATPases in these tissues.

While 74% of the H⁺-ATPases in *L. luymesii* plume tissue were P-type ATPases, 66% of the H⁺-ATPases in *S. jonesii* plume tissue were V-type ATPases (Table 2).

V-type H⁺-ATPases are important for creating proton gradients that drive secondary membrane transport across the plasma membranes of some invertebrates (Azuma and Ohta 1998; Wieczorek et al. 2000). In *R. pachyptila*, V-type ATPases are co-localized with the enzyme carbonic anhydrase on the apical region of the plume epithelium, and are thought to generate acidic extracellular pH conditions that facilitate CO₂ uptake (De Cian et al. 2003). V-type H⁺-ATPases represent on average 24% of the total ATPase activity in *S. jonesii* plume tissue (Fig. 3), whereas they represent only 14% of the total ATPase activity in *R. pachyptila* plumes (Goffredi and Childress 2001).

The unusually high abundance of V-type H⁺-ATPases in the *S. jonesii* plume tissue might indicate that this tubeworm relies heavily on its plume for CO₂ uptake. In contrast, V-type H⁺-ATPases represent only 10% of the total ATPase activity in *L. luymesii* plume tissue (Fig. 3). A previous study suggested that *L. luymesii* might supplement plume CO₂ uptake with HCO₃⁻ uptake across their roots (Dattagupta et al. 2006). The results of this study are consistent with this possibility.

Possible caveats of this study and future directions

In this study, we used an in vitro approach in which we exposed tissue homogenates to various inhibitors to deduce membrane transport mechanisms. While inhibitors are powerful tools for arriving at mechanistic deductions, lack of inhibitor sensitivity does not indicate the absence of a target protein (Cabantchik and Greger 1992). Moreover, differences in purification efficacy of membrane fractions could lead to artifacts. In our study, we found plasma membrane purification efficiencies were slightly higher for *L. luymesii* tissues than *S. jonesii* tissues, which could lead us to underestimate *S. jonesii* ATPase activities. Our study provides the first clues to the membrane transport mechanisms used by seep tubeworms for proton elimination. Future studies using purified membrane vesicles, or tissue localization with specific antibodies (De Cian et al. 2003) could provide more conclusive evidence for the existence of specific membrane proteins such as proton channels in root tissues of seep tubeworms.

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